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Siderophore Production and DNA Hybridization Groups of *Aeromonas* spp.

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A correlation between the genospecies (DNA-DNA hybridization group) and the type of siderophore produced by 118 isolates of the genus *Aeromonas* was established. Organisms in hybridization groups 1 through 5 (including 5A, 5B, and 5AB) and group 12 predominantly produced the siderophore amonabactin, while an enterobactinlike siderophore was prevalent in groups 8/10 and 9. The siderophore produced by strains in group 6 may be an as-yet-unidentified nonphenolate, nonhydroxamate compound, and group 7 isolates synthesized no siderophores. Determination of the indigenous siderophore (or the absence of one) produced by an isolate of the genus *Aeromonas* may assist in identification of the organism's genetic species and may suggest the presence of certain virulence properties.

Siderophore-mediated iron acquisition is one of the ways bacteria and fungi obtain iron from many environments, including the vertebrate hosts of these microorganisms (4, 5, 9, 10, 12, 14, 20, 21, 24, 28, 29). Although siderophores are structurally diverse, most (but not all) are secondary hydroxamates or phenolates (usually derivatives of 2,3-dihydroxybenzoic acid) and are of low molecular mass (about 400 to 1,000 Da). Siderophore-dependent iron uptake systems consist of two elements, the ferric chelating siderophore itself and a cognate cell-associated apparatus that processes the ferrisiderophore for delivery of the metal to metabolism. The cell-associated uptake components (which include a membrane-embedded ferrisiderophore receptor) are responsible for specificity; the lack of a receptor for a siderophore probably indicates that it is not used (21). Consequently, a microorganism usually maintains external receptors for its homologous siderophore and for one or more heterologous siderophores made by other microorganisms. The siderophore production and utilization profile of a species or strain may be unique, and these traits have been used to differentiate some members of the family *Enterobacteriaceae* (25).

Members of the gram-negative genus *Aeromonas* are waterborne bacteria found in both fresh and brackish waters (17). These organisms are pathogens of animals ranging from fish to humans; they are possible etiologic agents in intestinal and extraintestinal diseases (1). On the basis of biochemical characteristics, *Bergey's Manual of Systematic Bacteriology* recognizes four *Aeromonas* species: mesophilic *A. hydrophila*, *A. caviae*, and *A. sobria* and psychrophilic *A. salmonicida* (23). Since the publication of the 1989 edition, several new species (including *A. media*, *A. eucrenophila*, *A. schubertii*, *A. veronii*, "*A. jandaei*," and "*A. trota*") have been proposed (Table 1). There are at least 14 DNA hybridization groups; more than one group exists in some of the phenotypic species (Table 1), a result that suggests the taxonomy and classification of this microbial complex are incomplete (1, 6, 7, 16, 18). In addition, not all of the genospecies (hybridization groups) can be separated phenotypically (1). Recent work suggests that multilocus enzyme electrophoretic analyses and rRNA gene restriction patterns may be useful as taxonomic tools for the genus *Aeromonas* (19).

Most isolates of *Aeromonas* species produce a siderophore, either amonabactin or the enteric siderophore enterobactin (2, 3). Amonabactin is the most prevalent siderophore in the *A. hydrophila* phenospecies, while most *A. sobria* isolates produce enterobactin (3). Moreover, amonabactin-producing isolates are unable to use enterobactin and vice versa (3). Amonabactin may be an aeromonad virulence factor. Amonabactin-producing strains acquire iron from the vertebrate serum component Fe-transferrin, whereas isolates that synthesize enterobactin cannot utilize Fe-transferrin in serum as an iron source (20). This finding confirms results of earlier studies that showed that enterobactin is inactivated by albumin in serum (21). Both amonabactin- and enterobactin-producing isolates also can derive (by a siderophore-independent process) all necessary iron from the host's heme or hemoglobin (20). Strains that produce amonabactin may also display other virulence traits, because strains that synthesize amonabactin are more likely to be resistant to serum-complement lysis than are the enterobactin-producing strains (20). The present study determined whether synthesis of a specific siderophore, or the lack thereof, might cluster in certain DNA hybridization groups.

MATERIALS AND METHODS

Microorganisms. Fifty isolates of *Aeromonas* species were obtained from channel catfish and catfish ponds by the Food and Drug Administration, Biological Hazards Branch, Dauphin Island, Ala. One hundred eighty-four additional isolates were provided by S. Stuart and M. Carson, Melbourne, Australia; L. Pickering, University of Texas Medical Branch, Galveston; and J. Bertolini, Oregon State University Marine Science Center, Newport. These isolates were classified as phenospecies *A. hydrophila*, *A. caviae*, and *A. sobria* according to the schemes of Popoff (23) and Sakazaki and Balows (26). One hundred eighteen other isolates of the *Aeromonas* species (most isolated from human feces) for which the DNA hybridization groups had previously been

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TABLE 1. DNA hybridization groups and recognized and proposed species of the genus *Aeromonas*[a]

DNA hybridization group	Phenotypic species	Genetic species
1	*A. hydrophila*	*A. hydrophila*
2	*A. hydrophila*	
3	*A. hydrophila* *A. salmonicida*	*A. salmonicida* (various subsp.)
4	*A. caviae*	*A. caviae*
5A	*A. caviae*	*A. media* (subsp.)
5B	*A. caviae* *A. media*	*A. media* (subsp.)
6	*A. eucrenophila*	*A. eucrenophila*
7	*A. sobria*	*A. sobria*
8/10	*A. sobria* *A. veronii*	*A. veronii*
9	*A. sobria*	"*A. jandaei*"
11	*A. veronii*-like	
12	*A. schubertii*	*A. schubertii*
13	*A. schubertii*-like	*Aeromonas* group 501
14	"*A. trota*"	"*A. trota*"

[a] From references 1, 6, 7, 14, and 15.

determined (1) were included in the study. The amonabactin mutant *A. hydrophila* 201 (2) and the enterobactin mutant *Escherichia coli* AN90 (8) were provided by S. Barghouthi (University of Mississippi Medical Center, Jackson) and S. Stuart, respectively. All cultures were maintained in Luria broth or on tryptic soy agar plates (Difco Laboratories, Detroit, Mich.). The *Aeromonas* species cultures were incubated at 30°C, and the *E. coli* culture was grown at 37°C. Cultures were preserved frozen in 25% glycerol in nutrient broth (Difco) at −70°C.

Siderophore detection and identification. The chrome azurol S siderophore detection agar of Schwyn and Neilands (27), prepared as described by Barghouthi et al. (3), was used to detect siderophore production. Each isolate was transferred with a sterile wooden applicator to chrome azurol S agar as a single colony. Synthesis of a high-affinity iron chelator, such as a siderophore, yields a bacterial colony with an orange halo on chrome azurol S agar; isolates without a halo were classified as siderophore negative.

In order to identify the excreted siderophore, the cultures were grown in a low-iron, glucose–mineral salts medium, which derepressed the iron-regulated siderophore biosynthetic system (2). The medium was treated with Chelex 100 (to lower the amount of trace metal contamination) by previously described methods (3) and consisted of the following per liter of medium: glucose, 5 g; $(NH_4)_2HPO_4$, 1 g; K_2HPO_4, 4 g; KH_2PO_4, 2.7 g; magnesium, 830 μM; manganese, 40 μM; iron, 0.18 μM. The low-iron culture supernatants were assayed for the presence of a phenolate siderophore by assay for dihydroxy phenolates (13) and for a secondary hydroxamate siderophore by the Csaky assay (11). Production of amonabactin was determined by the procedure of Barghouthi et al. (3), in which the siderophore was partially purified by column chromatography on polyamide (ICN Biomedicals, Costa Mesa, Calif.), with methanol as the eluant. The eluate was chromatographed on thin-layer sheets of polyamide (F1700 from Schleicher & Schuell, Keene, N.H., or Cheng Chin from Accurate Chemical and Scientific Corp., Westbury, N.Y.). Butanol saturated with aqueous 1.7% ammonium acetate solution was the developing solvent; UV fluorescence and a 1% ferric chloride solution sprayed on the chromatograms allowed identification of the phenolates. Amonabactin is produced in two forms, one containing tryptophan (amonabactin T) and the other containing phenylalanine (amonabactin P). The presence of the two forms on the chromatograms was determined by comparison with authentic amonabactins T and P. Excretion of amonabactin was further confirmed by visualizing the tryptophan in amonabactin T with the Ehrlich reagent (3). To identify synthesis of an enterobactinlike siderophore, the low-iron culture supernatant (adjusted to a pH of 3 with 6 N HCl) was extracted with ethyl acetate. The ethyl acetate layer was chromatographed on cellulose plates (13255 from Eastman Kodak, Rochester, N.Y.) with a two-dimensional solvent system (22). Synthesis of an enterobactinlike siderophore was judged from the presence (detected by UV fluorescence) of intact enterobactin and its decomposition products (22) on the cellulose plates. Material resembling intact enterobactin was most readily demonstrated by extraction of 1 liter of culture supernatant that was in the early log phase of growth. To confirm the presence of the serine-containing siderophore enterobactin, the chromatographic spot representing intact enterobactin was scraped from the plates and the material was eluted with methanol. Amino acid analysis of this sample (done by the amino acid analysis laboratory, Department of Biochemistry, University of Mississippi Medical Center) identified serine as the only amino acid present.

A bioassay also was used to presumptively determine production of either amonabactin or the enterobactinlike siderophore. The amonabactin-requiring mutant *A. hydrophila* 201 and the enterobactin-requiring mutant *E. coli* AN90 were seeded into separate plates of Luria agar containing 250 μg of the chelating agent ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (Sigma Chemical Co., St. Louis, Mo.) per ml. The test isolate was then planted (with a sterile wooden applicator stick) as a colony on the surface of the solidified agar. Satellite colonies of the indicator strain around the test colony indicated the production of a utilizable siderophore by the test isolate. The absence of stimulation of the indicator strain was considered to be evidence for the lack of production of the appropriate siderophore. Since the full range of siderophores that can be used by the indicator strains is presently unknown, stimulation of the indicator strain only suggested production of a utilizable siderophore and did not represent a definitive identification of the siderophore produced.

RESULTS AND DISCUSSION

Previous studies (3) of siderophore synthesis by 44 isolates classified as phenospecies *A. hydrophila*, *A. caviae*, and *A. sobria* showed that more than 70% of the *A. hydrophila* isolates synthesized amonabactin, while about 20% may have produced enterobactin. Conversely, more than 80% of the *A. sobria* isolates probably synthesized enterobactin and few isolates produced amonabactin. In the initial surveys, only three *A. caviae* isolates were tested; all produced amonabactin. Present studies with an additional 234 isolates (classified as phenospecies) confirmed and extended these results (Table 2). The analyses revealed that, as with the *A. hydrophila* isolates, the majority (65%) of the *A. caviae* isolates were amonabactin producers. Among 104 additional strains of *A. sobria* that were tested, only three amonabactin producers were identified. Present studies also discovered strains that produced no detectable siderophore in each

TABLE 2. Siderophore type and phenotypic species in the genus *Aeromonas*

Species (no. of isolates)[a]	% (No.) of isolates producing:		
	Amonabactin	Enterobactinlike siderophore	No siderophore
A. hydrophila (104)	73 (76)	22 (23)	5 (5)
A. sobria (104)	3 (3)	81 (84)	16 (17)
A. caviae (26)	65 (17)	31 (8)	4 (1)

[a] Differential reactions described by Popoff (23) and Sakazaki and Balows (26) were used to establish phenospecies.

phenospecies; isolates producing no siderophore were most prevalent (16%) in the *A. sobria* phenospecies.

Each of the three major *Aeromonas* phenospecies encompasses more than one DNA hybridization group; therefore, it is possible that isolates producing either amonabactin, enterobactin, another siderophore type, or no detectable siderophore cluster in certain groups. To assess this possibility, the type of siderophore present in each of 118 *Aeromonas* species isolates that had previously been classified by DNA hybridization group (1) was determined. A correlation between the DNA group and the type of siderophore produced was apparent (Table 3). Of 39 isolates in groups 1 through 3 (phenospecies *A. hydrophila*), 35 produced amonabactin. One of the 19 isolates in group 1 may be an enterobactin producer. All but 1 of 45 isolates in groups 4, 5A, and 5B (phenospecies *A. caviae*) were amonabactin positive. The predominant siderophore made by groups 8/10 (phenospecies *A. sobria*) and 9 ("*A. jandaei*") resembled enterobactin. Three of the isolates included in group 8/10 were *A. veronii* CDC1169-83 (type strain), CDC1067-84, and CDC0715-84. As previously reported (3), none of the *A. veronii* strains produced amonabactin. The synthesis of siderophores by *A. veronii* strains CDC1169-83 and CDC0715-84 was marginal, and the material could be identified only as a phenolate; however, *A. veronii* CDC1067-84 produced a siderophore that was readily identifiable as a substance similar to enterobactin.

Aeromonad strains producing no apparent siderophore were present in several groups, and the highest number was in group 8/10. Both isolates of group 7 were siderophore negative; it is possible that group 7 is a siderophore-negative group. The *A. schubertii* group (group 12) appeared to be in the amonabactin-synthesizing category. Finally, a previously uncharacterized siderophore may have been discovered in group 6 (*A. eucrenophila*). Both group 6 organisms produced a siderophore (recognized by its reaction with the chrome azurol S siderophore detection system) that could not be chemically identified as a phenolate or a hydroxamate. Purification and characterization of this substance are in progress. The apparent lack of siderophore synthesis by group 7 and the production of an unusual siderophore by group 6 may somehow be related to the observation that strains in these groups have never been isolated from clinical specimens.

TABLE 3. Siderophore type and phenotypic species in the genus *Aeromonas*

DNA group (no. of isolates)	No. of isolates producing siderophore type			
	Amonabactin	Enterobactinlike	Uncharacterized	None
1 (19)	17	1	1[a]	
2 (10)	9		1[a]	
3 (10)	9			1
4 (17)	17			
5A (18)	17		1[a]	
5B (8)	8			
5A/B (2)	2			
6 (2)			2[b]	
7 (2)				2
8/10 (22)		16	2[a]	4
9 (4)		4		
11 (2)	1		1[a]	
12 (2)	2			

[a] Siderophore was a phenolate but did not resemble amonabactin or enterobactin on thin-layer chromatograms.

[b] Siderophore was not in the hydroxamate or phenolate category.

Although more strains in some of the groups should be tested to confirm our conclusions, the type of siderophore synthesized was sharply divided according to DNA hybridization group. For example, only one enterobactin-producing strain (in group 1) outside of groups 8/10 and 9 was found. Therefore, an isolate that produces enterobactin probably belongs to group 8/10 or 9. This is in contrast to data in Table 2 showing that 31% of the isolates phenotypically identified as *A. caviae* (groups 4 and 5) and 22% of the phenotypic *A. hydrophila* strains (groups 1 through 3) produced enterobactin. The discrepancies likely attest to the previously noted (17) inadequacy of biochemical schemes for the identification of *Aeromonas* species and reveal the more accurate correlation between hybridization groups and siderophores. No isolate that produced both amonabactin and the enterobactinlike siderophore was identified. Previous studies (20) showed that amonabactin can remove iron from host Fe-transferrin for microbial use; the enterobactinlike siderophore cannot do this in host serum. Aeromonad isolates producing either of these siderophores also can derive (by a siderophore-independent process) all necessary iron from host heme compounds. Whether or not amonabactin is an important virulence factor is not yet clear; however, amonabactin synthesis and the independent virulence trait of resistance to serum-complement lysis tend to occur together (20). The hypothesis that amonabactin producers have two separate routes for acquisition of host iron during an infection, whereas the strains producing enterobactin must rely on uptake of iron from heme, can be suggested. Present studies imply that, in combination with other phenotypic traits, the type of siderophore synthesized (or the absence of one) may be a useful characteristic in the separation of some of the genospecies of the genus *Aeromonas* and possibly in the evaluation of their potential virulence.

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